

**Appendix B:
"Marked Up" Version of Substitute Specification**

**MODEL FOR FUSION PROTEIN FUNCTIONING AS MUTUALLY EXCLUSIVE
DOMAIN FOLDING A MOLECULAR SWITCH**

Background of the Invention

[0001] The invention relates generally to a model for a mutually exclusive folding domain
molecular switch to modulate the bioactivity of proteins fusion protein.

1. Technical Field

2. Related Art

[0002] Ribonucleases are hydrolase enzymes that break linkages between nucleotides in ribonucleic acid. They are accordingly highly cytotoxic. A major problem with their use as therapeutic agents, for example, as pharmacologic agents in the treatment of cancer, is that their cytotoxicity is indiscriminate. Currently available ribonuclease pharmacologic agents kill normal as well as neoplastic cells, and the side effects of their use can be severe. Additionally, currently available ribonuclease agents demonstrate poor bioavailability owing to their rapid degradation by the liver and their difficulty in passing through both normal and neoplastic cell membranes.

Summary of the Invention

[0003] The present invention comprises a model for a mutually exclusive folding domain
molecular switch fusion protein (and a method for the creation thereof), which model
~~molecular switch includes comprises~~ a fusion protein comprising including includes at least
~~one~~ a ubiquitin insert protein having an insert regulatory domain lying between an amino
terminal and a carboxyl terminal of the ~~at least one~~ ubiquitin insert protein, the ubiquitin insert
protein being associated with a first quantity of free energy; and, a barcode target protein

having at least one a surface loop that begins at a first surface loop amino acid an alpha carbon of an initial amino acid of the surface loop and terminates at a second surface loop amino acid an alpha carbon of a terminal amino acid of the surface loop, the at least one surface loop comprising a target cytotoxic domain of the barnase target protein, the target cytotoxic domain being associated with a second quantity of free energy, wherein, the at least one ubiquitin insert protein is operatively inserted at a point within the at least one surface loop between the first surface loop amino acid alpha carbon of the first amino acid of the surface loop and the second surface loop amino acid alpha carbon of the terminal amino acid of the surface loop, such that an amino-carboxyl length extending between an alpha carbon of the amino terminal of the at least one insert protein and an alpha carbon of the carboxyl terminal of the at least one ubiquitin insert protein is at least two-times greater than an alpha carbon-alpha carbon length extending between the alpha carbon of the first surface loop amino acid and the alpha carbon of the second surface loop amino acid of the barnase target protein.

Brief Description of the Drawings

- [0004] FIG. 1A is a schematic illustration of an insert regulatory domain of an exemplary a ubiquitin insert protein in an unfolded conformation
- [0005] FIG. 1B is a schematic illustration of an insert regulatory domain of an exemplary a ubiquitin insert protein in an folded conformation.
- [0006] FIG. 1C is a schematic illustration of a conformation of an exemplary a barnase target protein having a folded target cytotoxic domain in the shape of a wedge and having a surface loop.
- [0007] FIG. 1D is a schematic illustration of a conformation of a barnase target protein having an unfolded target cytotoxic domain in the shape of a straight line and having a surface loop.
- [0008] FIG. 1E is a schematic illustration of a ubiquitin-barnase exemplary fusion protein capable

of existing in two mutually exclusive conformations.

[0009] FIG. 1F is a schematic illustration of a ubiquitin-barnase ~~exemplary~~ fusion protein capable of existing in two mutually exclusive conformations, in which an equilibrium state has been influenced by the binding of a ligand.

[00010] FIG. 2A is a schematic illustration of a human ubiquitin molecule.

[00011] FIG. 2B is a schematic illustration a barnase molecule.

[00012] FIG. 3 is a graph of circular dichroism spectra of a ubiquitin-barnase fusion protein as a function of temperature, showing temperature-induced conformational change in the structure of ubiquitin-barnase fusion protein.

[00013] FIG. 4 is a graph of the conversion an ubiquitin-barnase fusion protein from the barnase conformation to the ubiquitin conformation, monitored by ellipticity at 230 nm (open triangles, solid line) and barnase enzymatic activity (closed triangles, dashed line). Lines are for illustrative purpose only.

[00014] FIG. 5 is a graph showing the denaturation of the barnase domain (10 °C, circles) and the ubiquitin domain (40 °C, squares) induced by urea and guanidine hydrochloride ("GdnHCl"), respectively.

[00015] FIG. 6 is a graph of a circular dichroism spectral recording at 40 degrees C, showing barnstar-induced folding of the barnase domain and unfolding of the ubiquitin domain.

[00016] FIG. 7 is a graph of a circular dichroism spectral recording at 15 degrees C, showing barnstar-induced folding of the barnase domain and unfolding of the ubiquitin domain.

Detailed Description of the Invention

[00017] The model for a fusion protein of the present invention functions as a mutually exclusive domain folding domain molecular switch is ~~provides a novel and powerful approach~~ mechanism to ~~for~~ understanding the fundamental mechanisms of allosteric switching in molecular biology and for the developing diagnostic and therapeutic proteins with novel capabilities, possessing the following advantages:

- a) the mechanism of the ~~molecular switch~~ fusion protein is inherently cooperative; and,
- b) the all-or-nothing action of the switching mechanism of the ~~molecular switch~~ fusion protein assures that ~~it~~ the fusion protein behaves in a binary fashion; and,
- c) the switching mechanism of the fusion protein is reversible; and,
- d) the position of a reciprocally folding and unfolding conformational equilibrium of the fusion protein, to be described hereinafter, can be readily adjusted by external factors.

[00018] In their simplest form, proteins are polypeptides, i.e., linear polymers of amino acid monomers. However, the polymerization reaction which produces a polypeptide results in the loss of one molecule of water from each amino acid. Consequently, a polypeptide is more rigorously defined as a polymer of amino acid residues. Natural protein molecules may contain as many as 20 different types of amino acid residues, each of which contains a distinctive side chain.

[00019] An amino acid is an organic molecule containing an amino group ("NH₂") and a carboxylic acid group ("COOH"). While there are many forms of amino acids, all of the important amino acids found in living organisms are alpha-amino acids. Alpha amino acids

have their both their -COOH and -NH_2 groups attached to the same carbon atom, which is called the alpha carbon atom.

[00020] Thus, all of the important amino acids found in living organisms consist of an alpha carbon atom to which there is attached:

- a) A hydrogen atom;
- b) An amino group (-NH_2);
- c) A carboxyl group (-COOH);
- d) One of 20 different "R" groups.

[00021] It is the structure of the R group that distinguishes each amino acid structurally and determines its biochemical properties. Moreover, the structure and biochemical properties of a protein ~~is~~ are dictated by the precise sequence of the amino acids in the polypeptide chains of which it is comprised. One end of every polypeptide, called the amino terminal or N-terminal, has a free amino group (-NH_2). The other end, has a free carboxyl group (-COOH), and is called the carboxyl terminal or C-terminal.

[00022] The particular linear sequence of amino acid residues in the polypeptide chain comprising a protein defines the primary structure of that protein. However individual polypeptides and groups of polypeptides undergo spontaneous structural alteration and association into a number of recurring intermediate patterns such as, for example, helices, including alpha helices, and sheets, including beta sheets. These recurring intermediate polypeptide patterns are referred to as a protein's secondary structure. The spontaneous structural alteration and association of polypeptide chains into a secondary structure is determined by the sequence of amino acids in the polypeptide chains and by the ambient biochemical environment.

[00023] The helices, sheets, and other patterns of a protein's secondary structure additionally undergo a process of thermodynamically-preferred compound folding to produce a three-

dimensional or tertiary structure of the protein. The fully folded conformation of the protein is maintained by relatively weak inter-atomic forces such as, for example, hydrogen bonding, hydrophobic interactions and charge-charge interactions. Covalent bonds between sulphur atoms may also participate in protein folding into a tertiary conformation by forming intramolecular disulfide bridges in a single polypeptide chain, as well as by forming intermolecular disulfide bridges between separate polypeptide chains of a protein. This ability of polypeptide chains to fold into a great variety of structures, combined with the large number of amino acid sequences of a polypeptide chain that can be derived from the 20 common amino acids in proteins, confers on protein molecules their great range of biological activity.

[00024] The tertiary structure of a protein may contain a surface loop. As used herein, the term surface loop means is a continuous length of polypeptide chain whose constituent amino acids are in neither an alpha helical conformation nor in a beta sheet conformation, and can contact at least five water molecules, as determined by the DSSP computer program of Wolfgang Kabsch and Chris Sander. The DSSP, a program which is well known in the art, defines secondary structure, geometrical features and solvent exposure of proteins, given atomic coordinates in Protein Data Bank format, which is also well known in the art. (W. Kabsch & C. Sander, "Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical figures", Biopolymers 22, 2577-2637. (1983); See also, Centre for Molecular and Biomolecular Informatics, University of Nijmegen, Toernooiveld 1, P.O. Box 9010, 6500 GL Nijmegen, +31 (0)24-3653391, postmaster@cmi.kun.nl; <http://www.embl.kun.nl/gv/dssp/>)

[00025] Consistently with the foregoing definition, the term "surface loop" additionally means the cytotoxic domain of the barnase target protein, beginning at an alpha carbon of an initial amino acid of the surface loop and terminating at an alpha carbon of a terminal amino acid of the surface loop.

[00026] Protein folding occurs on a global level that endows the entire protein molecule with a

three dimensional structure and surface topology. Protein folding also occurs at a local level at multiple sites upon and within a protein. Locally, folding may involve one or more polypeptide subunits of the protein to endow different regions of the protein with different specific biological activities, or different specific molecular architectures, such as, for example, fashioning a location in a protein molecule into a receptor site for another molecule.

[00027] As used herein, the term domain means the molecular structure of an entire protein molecule or the molecular structure of a part, portion, or region of the molecular structure of a protein molecule, including a part, portion, or region of the protein molecule's surface or the protein molecule's interior. A domain may refer only to a distinction in a protein molecule's structure, such as for example, an alpha helix or a beta sheet. A domain may or may not have an associated biological function, such as a regulatory, receptor, signaling, active, catalytic, or other biological function. A domain may further be associated with a free energy, i.e., a thermodynamic state function that indicates the amount of energy that stabilizes the domain when the protein, or part thereof, with which the domain is associated is in a folded configuration. All of part of the free energy may be available for the domain to do biochemical work.

[00028] Because the folding of a protein molecule is both a global and local process, it can endow a protein molecule with both global and local structural and biological properties, such as, for example, an enzymatic activity, or a capacity and specificity for binding other proteins, such as antigens. Consequently, the biological functions of a protein depend on both its global folded tertiary structure, which is also called its native or folded conformation, as well as the folded structure of regions of the protein. Conversely, a global or local unfolding of a protein deactivates its global or local biological activity. An unfolded, biologically inactive protein is said to be in a denatured or unfolded conformation.

[00029] Many proteins are comprised of domains that communicate with each other by means of conformational changes in the structure of the protein of which they are a part, in order to

activate or deactivate a biological function. For example, in the case of a protein that is an enzyme, ligand binding or phosphorylation can serve as a switching mechanism to induce structural changes within the enzyme's regulatory domain, which then triggers activity in the enzyme's catalytic domain.

[00030] Another type of switching mechanism is illustrated in vivo by proteins that are unfolded in physiological conditions but fold upon binding to a cellular target. In this molecular switching mechanism, the folding and unfolding of a regulatory domain of a protein modulates the function of the protein via propagation of structural changes to its active domain.

[00031] ~~In the case of the a~~ The fusion protein herein serving as an exemplary embodiment of the model for {1stOA} a mutually exclusive domain folding (hereinafter, "MEDF") switch, the protein is an {1stOA} exemplary fusion protein that is synthesized from:

- a) an exemplary ubiquitin insert protein having an insert regulatory domain lying between an amino terminal and a carboxyl terminal of the ubiquitin insert protein, which insert regulatory domain is associated with a first quantity of free energy; and,
- b) an exemplary barnase target protein having ~~a~~ at least one surface loop that begins at an alpha carbon of a first surface loop amino acid an initial amino acid of the surface loop and terminates at an alpha carbon of a second surface loop amino acid, terminal amino acid of the surface loop, which surface loop comprises a target cytotoxic domain associated with a second quantity of free energy.

[00032] The amino terminal of the exemplary ubiquitin insert protein is spatially separated from the carboxyl terminal of the exemplary ubiquitin insert protein by a linear (i.e., straight line) distance known as the amino-carboxyl length (hereinafter, the "N-C terminal length") of the exemplary ubiquitin insert protein, that is measured when the exemplary ubiquitin insert

protein is in its folded conformation-; see, e.g. double-headed arrow in FIG. 2A. The first surface-loop amino acid alpha carbon of the initial amino acid of the surface loop of the exemplary barnase target protein is spatially separated from the second surface-loop amino acid alpha carbon of the terminal amino acid of the surface loop of the exemplary barnase target protein by a linear (*i.e., straight line*) distance known as the alpha-carbon-alpha-carbon length of the surface loop of the exemplary barnase target protein (hereinafter, the “Calpha-Calpha length”), that is also measured when the exemplary barnase target protein is in its folded conformation-; see, e.g. double-headed arrow in FIG. 2B.

[00033] The molecular structure of the exemplary ubiquitin-barnase fusion protein is engineered so that, at any time, the folding of the ubiquitin insert regulatory domain necessarily unfolds the barnase target cytotoxic domain, and vice versa, thereby making the folded and unfolded states of the insert regulatory and target cytotoxic domains mutually exclusive. This mutual exclusion of concurrently folded or concurrently unfolded states is accomplished by the insertion of the exemplary ubiquitin insert protein into the surface loop of the exemplary barnase target protein subject to a novel structural design criterion wherein the N-C terminal length of the exemplary ubiquitin insert protein is at least two-times greater than the Calpha-Calpha length of the surface loop of the exemplary barnase target protein.

[00034] The present invention is thus a fusion protein that functions as an exemplary embodiment of a model for a mutually exclusive domain folding (“MEDF”) a molecular switch comprising an exemplary two domain, bifunctional fusion protein, wherein the free energy released by the folding of the ubiquitin insert regulatory domain a first domain of the exemplary fusion protein drives the unfolding of the barnase target cytotoxic domain a second domain of the exemplary fusion protein, and vice versa.

[00035] Subject to this novel structural design criterion, a dynamic state of thermodynamic and structural equilibrium is established in the exemplary fusion protein that disengages the regulatory insert domain of the exemplary ubiquitin insert protein and the cytotoxic target

domain of the ~~exemplary barnase~~ target protein from simultaneously co-existing in their native folded states.

[00036] Accordingly, any excess free energy present in one of the two domains that is not necessary to stabilize its folded configuration is spontaneously transferred, through the structure of the ~~exemplary~~ fusion protein, to the other of the two domains to unfold it from its folded configuration, and vice versa. In effect, the excess free energy stored in the folded conformation of one domain is used to drive the unfolding of the other domain; and, the molecular structure of the ~~exemplary~~ fusion protein is engineered to create a dynamic state of thermodynamic and correlative structural equilibrium that is determined by the relative thermodynamic and structural stabilities of the two domains.

[00037] Viewed another way, the molecular structure of the ~~exemplary~~ fusion protein is engineered to create a ~~MEDF~~ molecular switch by creating cooperatively folding-unfolding subunits comprising two protein domains, which two domains cannot simultaneously exist in their folded states. This scheme is depicted in FIGS. 1A – F.

[00038] FIG. 1A shows a schematic illustration of a domain of an exemplary insert protein in an unfolded conformation. FIG. 1A is a schematic illustration of an insert regulatory domain of a ubiquitin insert protein in an unfolded conformation. In FIG. 1A, ~~exemplary~~ ubiquitin insert protein 51, having an amino terminal 21 and a carboxyl terminal 22, exists in an unfolded conformation 20, thereby forming an ~~exemplary~~ unfolded insert regulatory domain, schematically illustrated as a hatched ribbon that is coincident with the extent of ~~exemplary~~ ubiquitin insert protein 51.

[00039] FIG. 1B shows a schematic illustration of a domain of an exemplary insert protein in an folded conformation. FIG. 1B is a schematic illustration of an insert regulatory domain of a ubiquitin insert protein in an folded conformation. In FIG. 1B, ~~exemplary~~ ubiquitin insert protein 51, having an amino terminal 21 and a carboxyl terminal 22, exists in a folded

conformation 23, thereby forming an ~~exemplary~~ folded insert regulatory domain, schematically illustrated as a hatched double-crossed ribbon that is coincident with the extent of ~~exemplary~~ ubiquitin insert protein 51, and folds to form indentation 24.

[00040] In Fig. 1B, reference numeral 25 refers to the amino-carboxyl length of insert regulatory domain of insert protein barnase in its folded conformation (corresponding to double-headed arrow labeled 38 Å in FIG. 2A). The amino-carboxyl length of the insert regulatory domain of insert protein barnase in its folded conformation is synonymous with the N-C terminal length of the labeled 38 Å in FIG. 2A). The amino-carboxyl length of the insert regulatory domain of insert protein barnase in its folded conformation (also corresponding to double-headed arrow labeled 38 Å in FIG. 2A).

[00041] In FIG. 1C, there is shown schematically a folded conformation 26 of an ~~exemplary~~ barnase target protein 41 having an ~~exemplary~~ folded target cytotoxic domain in the shape of a wedge 46. Barnase ~~Exemplary~~ target protein 41 also has a surface loop 27, schematically shown as a nearly full circle, arising from ~~a first an alpha carbon of an initial amino acid of the surface loop,~~ forming a first terminus beginning 28 (of a first arm 29) of wedge 46, and ending at an second alpha carbon of a terminal amino acid of the surface loop, forming an second terminus end 30 (of a second arm 31) of wedge 46.

[00042] Also shown schematically in FIG. 1C is line 45, representing the (straight) C α -C α length of the surface loop 27.

[00043] In FIG. 1D, there is shown schematically an unfolded conformation 32 of ~~exemplary~~ barnase target protein 41 in which ~~exemplary~~ folded target cytotoxic domain 46 (of FIG. 1C) has unfolded into the shape of straight line 56. Unfolded conformation 32 of ~~exemplary~~ barnase target protein 41 also has surface loop 27, now shown as a half-circle arising from first the alpha carbon forming a the first terminus beginning 28 of the straight line 56 and ending at a second the alpha carbon forming a the second terminus end 30 of the straight line 56.

[00044] In FIG. 1E, there is shown schematically an exemplary ubiquitin-barnase fusion protein **35** comprised of including exemplary ubiquitin insert protein **51** inserted into surface loop **27** of exemplary barnase target protein **41**, which exemplary ubiquitin-barnase fusion protein **35** is capable of existing in two mutually exclusive conformations **35L** and **35R**, representing the mutually exclusive binary states of the ~~model for the MEDF molecular switch comprising~~ embodied in exemplary ubiquitin-barnase fusion protein **35**.

[00045] The image to the left of the antiparallel arrows **36** of FIG. 1E shows exclusive state **35L** of exemplary ubiquitin-barnase fusion protein **35**, wherein exemplary ubiquitin insert protein **51**, with its exemplary insert regulatory domain in unfolded (hatched ribbon) conformation **20**, (as shown in FIG. 1A), has been inserted into surface loop **27** of exemplary barnase target protein **41** with its exemplary target cytotoxic domain in its folded conformation **46**, (as shown in FIG. 1C). The image to the right of the antiparallel arrows **36** of FIG. 1E shows exclusive state **35R** of exemplary ubiquitin-barnase fusion protein **35**, wherein exemplary ubiquitin insert protein **51**, with its exemplary insert regulatory domain in its folded (hatched double-crossed ribbon) conformation **23**, (as shown in FIG. 1B), inserted into surface loop **27** of exemplary barnase target protein **41** with its exemplary target cytotoxic domain in its unfolded (straight line) conformation **56** (as shown in FIG. 1D).

[00046] In FIG. 1F, exemplary ubiquitin-barnase fusion protein **35** is again shown schematically existing in two mutually exclusive conformations **35L** and **35R**, representing the mutually exclusive binary states of the ~~model for the MEDF molecular switch embodied in~~ comprising exemplary-fusion protein **35**. However, the dynamic equilibrium existing between conformations **35L** and **35R** has been shifted to the right by the binding of exemplary ligand **40** to the indentation **24** of exemplary insert regulatory domain of protein **51** in folded conformation **23**.

[00047] If the exemplary insert regulatory domain of exemplary ubiquitin insert protein **51** in folded conformation **23** (FIG. 1B and FIG. 1E Right) is more stable than exemplary target

cytotoxic domain ~~having wedge 46~~ of exemplary barnase target protein 41 in its folded conformation 26, (FIG. 1C and FIG. 1E Left), then exemplary insert regulatory domain of exemplary ubiquitin insert protein 51 in folded conformation 23 (FIG. 1B and FIG. 1E Right) will have an excess of free energy with which to forcibly stretch and unfold folded conformation 26 of exemplary target cytotoxic domain, ~~having wedge 46~~, of exemplary barnase target protein 41 (FIG. 1C and FIG. 1E Left), thereby unfolding wedge 46 into line 56, and yielding exemplary ubiquitin-barnase fusion protein 35 in state 35R.

[00048] If exemplary target cytotoxic domain, ~~having wedge 46~~, of exemplary barnase target protein 41 in folded conformation 26 (FIG. 1C and FIG. 1E Left) is more stable than exemplary insert regulatory domain of exemplary ubiquitin insert protein 51 in folded conformation 23 (FIG. 1B and FIG. 1E Right), then exemplary target cytotoxic domain, ~~having wedge 46~~, of exemplary barnase target protein 41 in its folded conformation 26 (FIG. 1C and FIG. 1E Left) will have an excess of free energy with which to forcibly stretch and unfold exemplary insert regulatory domain of exemplary ubiquitin insert protein 51 in folded conformation 23 (FIG. 1B and FIG. 1E Right), thereby folding line 56 into wedge 46, and yielding exemplary ubiquitin-barnase fusion protein 35 in state 35L.

[00049] In this manner, ~~exemplary embodiment of the model for a~~ the ubiquitin-barnase fusion protein ~~MEDF molecular switch~~ fully exploits the free energy stored in the folded conformations of the aforementioned domains, as well as the inherent cooperativity of reciprocal domain folding, to create a molecular switch of unprecedented efficiency. Consequently, ~~the~~ The model for a MEDF molecular switch ubiquitin-barnase fusion protein is a novel and powerful approach to understanding the fundamental mechanisms of allosteric switching in molecular biology and for the developing diagnostic and therapeutic proteins with novel capabilities, possessing the following advantages:

- a) the mechanism of the molecular switch it is inherently cooperative; and,
- b) the all-or-nothing action of the mechanism of the molecular switch assures

- that it behaves in a binary fashion; and,
- c) the switching mechanism is reversible; and,
- d) the position of the folding/unfolding equilibrium can be readily adjusted by external factors.

[00050] For example, When present at 1 mM concentration, a ligand that binds to a protein with a dissociation constant of 1 nM, will stabilize the native conformation of the protein by as much as $RT \ln(10^3)$, or 4.2 kcal mol⁻¹ at 37 degrees C. This value is comparable to the total free energy change of folding for many proteins.

[00051] As indicated, barnase, is a highly cytotoxic ribonuclease that breaks linkages between nucleotides in ribonucleic acid. A major problem with its use as a pharmacologic agent, e.g., in the treatment of cancer, is that its cytotoxicity is indiscriminate. Currently available ribonuclease pharmacologic agents, such as barnase, kill normal as well as neoplastic cells, and the side effects of their use can be severe.

[00052] Because, the regulatory domain of ubiquitin and the cytotoxic domain of barnase cannot simultaneously co-exist in their folded states, the regulatory domain of ubiquitin, may be used to regulate the cytotoxic activity of barnase. Moreover, the ubiquitin and barnase domains participate in a cooperative and reversible conformational equilibrium, that may be influenced and controlled by a variety of controllable effector signals such as, for example, ligand binding, pH, temperature, chemical denaturants, or the presence of stabilizing or destabilizing mutations in either the barnase or ubiquitin domains.

[00053] ~~While the exemplary embodiment of the model for a [1stOA] MEDF-switch entails the creation of a~~ The two-domain, bifunctional exemplary ubiquitin-barnase fusion protein to be described more fully hereinafter, ~~the exemplary embodiment of the model for a [1stOA] MEDF-switch disclosed herein is not limited to the insertion of an exemplary ubiquitin insert protein into an exemplary barnase target protein having only one domain or only one~~

biological function. The ~~exemplary embodiment of the model for a MEDF switch~~ two-domain, bifunctional ubiquitin-barnase fusion protein disclosed herein comprises cases wherein one or more exemplary insert proteins is inserted into one or more surface loops of exemplary target proteins having multiple domains and multiple biological functions, the effect of these insertions being to form a one or more cooperatively folding-unfolding subunits in the resultant ~~exemplary~~ fusion protein, each comprising two protein domains, which two domains cannot simultaneously exist in their folded states, thereby forming one or more cooperative, reversible, MEDF molecular switches in the same ~~exemplary~~ fusion protein, each of which is responsive to different controllable effector signals such as, for example, ligand binding, pH, temperature, chemical denaturants, or the presence of stabilizing or destabilizing mutations in either the ~~barnase or ubiquitin~~ domains.

[00054] To create ~~a specific nonlimiting exemplary embodiment of the model for a MEDF switch,~~ the inventors herein created a the two-domain bifunctional ubiquitin-barnase fusion protein, ~~the inventors herein inserted comprising the insertion of an exemplary insert protein,~~ the human ubiquitin molecule having one regulatory domain, into a selected surface loop of ~~an exemplary the barnase~~ target protein, ~~barnase~~, which surface loop has one catalytic (or cytotoxic) domain.

[00055] The exemplary insert protein, human ubiquitin, is a protein having one regulatory domain, and one biological function, that of serving as a signaling marker or flag.

[00056] The exemplary target protein, barnase is a ribonuclease produced exclusively by the bacterium *Bacillus amyloliquefaciens*. Barnase has one catalytic domain that is functionally cytotoxic to all mammalian cell types.

[00057] FIG. 2A is a schematic illustration a molecule of human ubiquitin. The sheet-like arrows and ribbons in FIG. 2A represent beta strands and alpha helices, respectively. The noodle-like strands in the protein are loops and turns of the ubiquitin molecule. In FIG. 2A, the N-C

terminal length from ubiquitin is about 38 Å in its folded conformation, as indicated by the double-headed straight-line arrow bearing the legend “38 Å”, a magnitude that measures the straight-line distance between an N-terminal amino acid at which one arrowhead begins and a C-terminal amino acid, at which the opposing arrow head ends.

[00058] FIG. 2B is a schematic illustration a barnase molecule. The sheet-like arrows and ribbons in FIG. 2A represent beta strands and alpha helices, respectively. The noodle-like strands in the protein are loops and turns of the barnase molecule. The barnase molecule has a surface loop in which the Calpha-Calpha length, measured from alpha-carbon of the loop amino acid proline in the number 64 position (“Pro64”) to the alpha-carbon of the loop amino acid threonine in the number 70 position (“Thr70”) is about 10.4 Å, with the barnase molecule in its folded conformation, as indicated by the double-headed straight-line arrow bearing the legend “10.4 Å”, a magnitude that measures the straight-line distance between an alpha carbon at which one arrowhead begins, and another alpha carbon, at which the opposing arrow head ends. The asterisk in FIG. 2B represents the point at which the ubiquitin molecule, shown in FIG. 2A is inserted between amino acid residue 66 and amino acid residue 67 of barnase.

[00059] The exemplary insert protein ubiquitin and the exemplary target protein barnase satisfy the novel structural design criterion that the straight line N-C terminal length of the exemplary insert protein be at least twice the straight line Calpha-Calpha length of the exemplary target protein surface loop selected for insertion. In the exemplary fusion protein resulting from the insertion of the exemplary insert protein ubiquitin molecule into the surface loop of the exemplary target protein barnase, the foregoing structural design criterion is satisfied.

[00060] Consequently, the regulatory domain of ubiquitin and the catalytic domain of barnase cannot simultaneously co-exist in their folded states; and, the regulatory domain of ubiquitin, may be used to regulate the cytotoxic activity of barnase. Moreover, the ubiquitin and barnase domains participate in a cooperative and reversible conformational equilibrium, that may be influenced and controlled by a variety of controllable effector signals such as, for example,

ligand binding, pH, temperature, chemical denaturants, or the presence of stabilizing or destabilizing mutations in either the barnase or ubiquitin domains.

[00061] The ubiquitin and barnase genes are created by annealing and ligating synthetic oligonucleotides (Integrated DNA Technologies) according to standard protocols.

[00062] The ubiquitin-barnase fusion gene is made by first adding an exemplary five amino acid linker (Gly-Thr-Gly-Gly-Ser) between the Lys66 and Ser67 codons of the barnase gene. The inserted DNA contains exemplary KpnI and BamHI restriction sites that are used to introduce the ubiquitin gene.

[00063] The exemplary five amino acids of the exemplary linker individually serve as short, flexible linkers at the points of attachment. The ubiquitin gene is inserted between the Thr and Gly codons of the linker.

[00064] All genes are fully sequenced to verify their integrity.

[00065] An interim ubiquitin-barnase fusion expression plasmid pETMT is created by using exemplary NdeI and XhoI enzymes to insert the ubiquitin-barnase fusion gene into a plasmid, such as, for example, a pET25b(+) plasmid (Novagen), or any other T7 promotor-containing plasmid that also confers resistance to an antibiotic other than ampicillin.

[00066] In order to make the plasmid stable in *E. coli*, the gene for barstar, the intracellular inhibitor of barnase that is co-expressed with barnase by *Bacillus amyloliquefaciens* (together with its natural promoter from *Bacillus amyloliquefaciens*), is cleaved out of an exemplary pMT1002 plasmid (gift of Dr. Y. Bai, National Institutes of Health), or any other T7 promotor-containing plasmid that also confers resistance to an antibiotic other than ampicillin, with ClaI and PstI enzymes. The barstar gene is then placed between ClaI and PstI restriction sites on the pETMT plasmid (prior to this step, these sites are introduced using the QuikChange

mutagenesis kit (Stratagene)).

[00067] In order to obtain milligram quantities of the ubiquitin-barnase fusion protein, it is necessary to increase cellular levels of barstar and purify the inactive ubiquitin-barnase fusion-barstar complex. Accordingly, the barstar gene is cloned into an exemplary pET41 plasmid (Novagen), thereby placing it under control of a T7 promoter and conferring upon the transformed cells resistance to kanamycin or any other antibiotic other than ampicillin.

[00068] *E. coli* BL21 (DE3) cells are transformed with both plasmids, grown in a temperature range between about 20 degrees C and 37 degrees C in exemplary Luria-Bertani medium containing ampicillin and kanamycin to $OD_{600} = 1.0$, and induced with 100 mg/L IPTG. Bacteria are harvested about 2 to 12 hours later by centrifugation.

[00069] Cells are lysed in about 10 mM sodium phosphate (pH 7.5) by repeated freeze-thaw cycles in the presence of a small amount of lysozyme at a concentration of about lysozyme is 10 mg/liter. Exemplary DNase I (Sigma) at a concentration of about 10 mg/liter is then added to reduce viscosity, and the solution is centrifuged to remove insolubles. 8 M urea is added to the supernatant to dissociate bound barstar, which is subsequently removed by passing the solution through DE52 resin (Whatman) or a substantially equivalent anion exchange chromatography resin. The solution is then loaded onto a HiTrap heparin column (Amersham-Pharmacia) or substantially equivalent cation exchange column, washed with 10 mM sodium phosphate (pH 7.5) and 6 M urea, and eluted with a 0-0.2 M NaCl gradient.

[00070] Western blot analysis using anti-ubiquitin antibodies is used to show that the major impurities are truncated ubiquitin-barnase fusion protein products in which the ubiquitin domain, which is unfolded in the ubiquitin-barnase fusion protein-barstar complex, is partially digested. These proteins, however, elute significantly later than the intact ubiquitin-barnase fusion protein in the NaCl gradient. The urea is removed by dialysis against double-distilled water, to yield barnase-ubiquitin fusion protein that is approximately 95% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

[00071] To confirm the switching mechanism of the ~~model for the MEDF~~ molecular switch as embodied in the exemplary ubiquitin-barnase fusion protein, and to characterized its structure, stability, and enzymatic function, experiments were performed by the inventors herein upon the purified ubiquitin-barnase fusion protein, obtained as described hereinabove

[00072] All experiments were performed in 10 mM potassium phosphate (pH 7.5), 0.1 M NaCl. The circular dichroism ("CD") spectra are shown in FIG. 3 as a function of temperature. In FIG 3., circles and squares indicate 5 °C and 50 °C, respectively; other scans were recorded at 5 °C increments between these two limits. Below 20 °C, the barnase-ubiquitin fusion protein exhibits molar ellipticities and spectral features nearly identical to those of barnase. A particularly diagnostic characteristic of barnase that is also observed for the barnase-ubiquitin fusion protein is a minimum at 231 nm attributed to Trp94. As temperature is increased, however, the spectrum shifts to one that strongly resembles ubiquitin. The 231 nm minimum disappears, and the position and molar ellipticity of the new minimum are consistent with native ubiquitin.

[00073] FIG. 4 shows a graph of the conversion a ubiquitin-barnase fusion protein from the barnase conformation to the ubiquitin conformation, monitored by ellipticity at 230 nm (open triangles, solid line) and barnase enzymatic activity (closed triangles, dashed line). Lines are for illustrative purpose only. Activities were determined by recording initial velocities in triplicate (2 mM ubiquitin-barnase fusion protein, 50 mM guanylyl(3'-5')uridine 3'-monophosphate ("GpUp")) and are normalized to the largest value. As can be seen from FIG. 4, the transition is fully reversible and has a midpoint of about 30 degrees C. Further, it appears to be two-state; an isodichroic point near 223 nm is apparent, and plotting the transition at different wavelengths yields identical midpoints.

[00074] The temperature-induced transition from barnase to ubiquitin is consistent with the higher thermal stability of ubiquitin. Ubiquitin and barnase unfold with midpoints of about 100

degrees C (pH 7) and 55 degrees C (pH 6.3) respectively. The possibility that the ubiquitin and barnase domains are simultaneously folded is ruled out by the observation that the molar ellipticity of the ubiquitin-barnase fusion protein never exceeds that of the individual proteins at any wavelength or temperature.

[00075] To further test the mutual exclusivity of ubiquitin and barnase domain folding, the inventors herein monitored urea-induced denaturation by Circular dichroism and Trp fluorescence. Ubiquitin and barnase contain zero and three Trp residues, respectively. Fluorescence is therefore expected to report primarily on structural changes within the barnase domain, whereas Circular dichroism reports on both domains. FIG. 5 is a graph showing the denaturation of the barnase domain (10 degrees C, circles) and the ubiquitin domain (40 degrees C, squares) induced by urea and guanidine hydrochloride ("GdnHCl"), respectively. Data were collected by Circular dichroism at 230 nm (open symbols, black lines) or by Trp fluorescence at 320 nm (closed symbols, grey line). Lines represent best fits to the linear extrapolation equation. At 10 degrees C, the Circular dichroism and fluorescence curves reveal a single cooperative unfolding transition. It appears to be two-state; thermodynamic parameters obtained by fitting both data sets to the linear extrapolation equation are identical within error ($ΔG = 4.1 ± 0.2 \text{ kcal} \times \text{mol}^{-1}$, $m = 2.2 ± 0.1 \text{ kcal} \times \text{mol}^{-1} \times \text{M}^{-1}$, $C_m = 2.3 ± 0.05 \text{ M}$). The fact that only one transition is apparent by Circular dichroism confirms that only one domain is folded. The agreement between Circular dichroism and fluorescence curves indicates that this domain is barnase.

[00076] If the situation is reversed by raising temperature above 30 degrees C, addition of denaturant at 40 degrees C is predicted to generate an unfolding transition by Circular dichroism but no transition by fluorescence. 6 M urea failed to produce a change in either spectrum. Addition of the stronger denaturant guanidine hydrochloride, however, yields the expected Circular dichroism unfolding transition with the following thermodynamic parameters: $ΔG = 8.5 ± 0.1 \text{ kcal} \times \text{mol}^{-1}$, $m = 2.5 ± 0.1 \text{ kcal} \times \text{mol}^{-1} \times \text{M}^{-1}$, $C_m = 3.4 ± 0.05 \text{ M}$, as shown in FIG. 5.

[00077] In contrast to molar ellipticity, fluorescence emission at 320 nm does not change significantly as a function of guanidine hydrochloride concentration, suggesting that the barnase Trp residues are solvent exposed at all denaturant concentrations. This conclusion is supported by the finding that the wavelength of maximum emission remains constant at 356 nm, the value for unfolded barnase. At 10 degrees C, this wavelength shifts from 340 nm in the absence of denaturant to 356 nm in 6 M urea. These data prove that:

- a) folding of the barnase domain induces unfolding of the Ubiquitin domain, and vice versa; and,
- b) temperature provides an efficient switch between the two folded conformations of the ubiquitin-barnase fusion protein.

[00078] To determine how tightly folding of the ubiquitin domain is coupled to unfolding of the barnase domain, the inventors herein measured the enzymatic activity of the ubiquitin-barnase fusion protein as a function of temperature, using the substrate guanylyl(3'-5')uridine 3'-monophosphate. As shown in FIG. 4, loss of The barnase-ubiquitin fusion protein activity in the ubiquitin-barnase fusion protein mirrors the structural conversion from barnase to ubiquitin, although the apparent midpoint of the former transition occurs at a higher temperature.

[00079] A likely reason is that substrate binding in the enzyme assays preferentially stabilizes the barnase domain. The complete loss of barnase activity above 50 degrees C, together with the molar ellipticity values shown in FIG. 3, provides strong evidence that ubiquitin domain folding induces complete unfolding of the barnase domain.

[00080] The preceding experimental results suggested to the inventors herein that temperature can be used to regulate cytotoxicity of the ubiquitin-barnase fusion protein in vivo. The inventors herein tested this prediction by transforming *E. coli* with a plasmid containing the ubiquitin-

barnase fusion protein gene under control of an isopropyl β -D-thiogalactopyranoside ("IPTG")-induced T7 promoter. Like barnase, the ubiquitin-barnase fusion protein is extremely lethal. The plasmid was found to be unstable in all strains of *E. coli*, including those that do not harbor the T7 RNA polymerase gene. Very few transformants were consistently recovered, and in each case the ubiquitin-barnase fusion protein gene was found to contain frameshift or nonsense mutations in the barnase coding region.

[00081] To overcome this problem, the inventors herein inserted the barstar gene and its natural promoter from *Bacillus amyloliquifaciens* into the foregoing plasmid, thereby creating a pETMT plasmid that is stable in *E. coli*.

[00082] As shown in Table 1 herein below, wherein figures are the averages with standard deviations are obtained from five plates, far fewer colonies were obtained when plates were grown at 15 degrees C compared to 37 degrees C. Moreover, 36 mg/m L IPTG was sufficient to kill nearly all of the bacteria at 15 degrees C, whereas 143 mg/mL IPTG was required to achieve a comparable result at 37 degrees C.

TABLE 1
Cytotoxicity of the barnase-ubiquitin fusion protein as a function of temperature and isopropyl β -D-thiogalactopyranoside concentration

Number of colonies at indicated concentration of IPTG						
T (°C)	0 mg/mL	9 mg/mL	18 mg/mL	36 mg/mL	72 mg/mL	143 mg/mL
15	14 \pm 5	35 \pm 18	39 \pm 4	0.3 \pm 0.5	0 \pm 0	0 \pm 0
37	160 \pm 40	250 \pm 60	257 \pm 40	274 \pm 30	12 \pm 4	0.60 \pm 1

[00083] These findings reflect the temperature-induced conformational shift from the barnase-form of the ubiquitin-barnase fusion protein to the ubiquitin form. Further, they demonstrate that the ubiquitin-barnase fusion protein/*E. coli* system is highly responsive to the relative

stabilities of the two domains within the temperature range of bacterial growth.

[00084] “Natively-unfolded” proteins contain subunits that are unstructured or partially structured in physiological conditions. The free energy of folding is provided by ligand binding interactions, and the resulting conformational change is used to modulate function of distant domains. The ubiquitin-barnase fusion protein captures this property as well. When barstar is added to the ubiquitin to form of ubiquitin-barnase fusion protein at 40 degrees C, a conformational change is observed. FIG. 6 is a graph of a circular dichroism spectral recording at 40 degrees C, showing barstar-induced folding of the barnase domain and unfolding of the ubiquitin domain.. Dotted lines represent 10 mM The barnase-ubiquitin fusion protein in the absence and presence of 12.5 mM barstar, respectively. Black lines were generated by subtracting the circular dichroism spectra of free barstar.

[00085] The circular dichroism spectrum of the ubiquitin-barnase fusion protein-barstar complex, after subtracting the spectrum of free barstar, resembles that of the ubiquitin-barnase fusion protein at 15 degrees C. FIG. 7 is a graph of a circular dichroism spectral recording at 15 degrees C, showing barstar-induced folding of the barnase domain and unfolding of the ubiquitin domain. Dotted lines represent 10 mM The barnase-ubiquitin fusion protein in the absence and presence of 12.5 mM barstar, respectively. Black lines were generated by subtracting the circular dichroism spectra of free barstar. The two are not identical, apparently because of minor structural differences between the free and bound states of barnase and/or barstar. However, Circular dichroism spectra of the ubiquitin-barnase fusion protein-barstar complexes at 40 °C and 15 °C are virtually indistinguishable. This result signifies that ubiquitin domain.

[00086] Accordingly, the inventors herein have demonstrated that the folding free energy of one protein domain can be used to drive unfolding of another. Because folding is reversible and inherently cooperative, this mechanism constitutes an efficient and responsive model for a mutually exclusive domain folding molecular switch for, inter alia, coupling conformational

change to protein function. The cytotoxic activity of barnase in the present invention provides the basis for several novel applications. For example, stability-enhanced ubiquitin variants can be rapidly identified from combinatorial libraries by their ability to allow *E. coli* to survive at temperatures less than 30 degrees C in the presence of 36 mg/mL IPTG.

[00087] A major advantage that this ~~model mutually exclusive domain folding molecular switch~~ fusion protein affords over other directed evolution techniques, such as, for example, phage display, is that the entire selection takes place inside a living bacterium, and stabilizing mutations are sorted from destabilizing mutations in the most efficient and decisive manner possible. This ~~model mutually exclusive domain folding molecular switch~~ fusion protein is general and can be applied to other proteins, including those too large to be expressed on the phage surface. If necessary, known stabilizing or destabilizing mutations can be introduced into the barnase domain in order to make the target protein optimally responsive to the inserted protein. Additionally, the ~~model mutually exclusive domain folding molecular switch~~ fusion protein can be modified to generate a class of cytotoxic molecules with novel sensor capabilities. For example, the ~~model mutually exclusive domain folding molecular switch~~ fusion protein allows ribonuclease activity to be turned on and off by ligand binding to an engineered regulatory domain. Ligand binding domains from any one of a large number of proteins can perform this function as long as they meet the structural design novel described hereinabove. This invention forms the basis for developing cytotoxic proteins that are activated by a wide variety of cell-specific effector molecules, and can thus target cancerous or virally infected cells for destruction.